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# Exposure Assessment of Aflatoxin M1 Through Ingestion of Infant Formula in Türkiye

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ABSTRACT

The objectives of the study were to determine the aflatoxin M1 (AFM1) content in ınfant formula samples in Türkiye and to assess the risk of infants' exposure to aflatoxins food contamination. A total number of 72 samples of infant formulas (premature, hipoallergenic, 0-6, 6-9,9-12 and 12-36 months) were analyzed for the presence of AFM1 using the Enzyme Linked Immunosorbent Assay (ELISA) method. 49% of the samples (35 samples) were contaminated with AFM1 and the group most highly contaminated with AFM1 was infant formulas 12-36 (8 samples) months. In addition, this study aimed at evaluating AFM1 levels especially above international (European Commission) (0.025 µg/kg) and local (Türkiye Food Codex Regulation) (0.025 µg/kg) standards and cancer risks associated with the ingestion of infant formula sampled from Türkiye. Five samples of infant formula had AFM1 concentration above maximum allowable concentration according to the standards. Risk assessments of AFM1 for infants ranged between 0.002- 0.035 ng/kg bw/day and >100.000–5263.15 respectively for Estimated Daily Intake (EDI), and Margin of Exposure (MOE). The riskiest group was determined in the 9-12 months samples. Results of our study indicate the high risk of infants' exposure, who are at the early stage of development and vulnerable to toxic contaminants.

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## Introduction

Infants and young children are the most sensitive to aflatoxins because of their greater relative exposure, undevelopment metabolism, and elimination, and higher growth and development rates (Mir et al., 2021). Also, aflatoxins toxicity is impacted by some nutritional agents. For example, babies with protein malnutrition are presumably more precision to aflatoxins (Coppa et al., 2019).

Milk-based and Cereal-based baby foods are the most serious nutrition source in infants and young children, mainly after 6 months of their birth (Sarmast et al., 2021, Demir and Demir, 2021). Infant formulas' components consist of milks (goat, cow) and different types of cereals (wheat, corn, oats, rice, barley, malt, and soy, and rye), honey, sugar, cocoa, and dried fruits (Khodaei et al., 2021). They are considered the best source of nutrition for the baby after or besides breastfeeding for the following reasons:

- Milk-based and cereal-based foods are important sources of energy supply (Agostoni et al., 2008), providing plenty of starch, fiber, and protein, besides high amounts of vitamins, minerals, and bioactive compounds (Fardet, 2010, Demir and Ağaoğlu 2021);
- They are vehicles for iron enrichment, also containing indigestible carbohydrates that play an important role in increasing the intestinal microbiota population (when infants are weaned, cereal feeding increases the fermentation activity of the gut microbiota) (Finn et al., 2017, Demir and Akpınar, 2021),
- These products have a mild taste, texture and semi-solid consistency, so it is the best alternative for babies in the transition phase from breast milk to solid foods at the beginning of complementary feeding (Sakashit at al., 2003). Despite all the mentioned advantages, prevalence and exposure to aflatoxins due to these products' consumption is inevitable (Hernandez et al., 2010).

Exposure of infants to AFM1 is important because the agent is group 2B probable carcinogenic by the International Agency for Research on Cancer (IARC, 2002). Chronic exposure of children to AFM1 causes malnutrition, liver cancer, low body weight, and low development in infancy and later life. Aflatoxins; They are secondary metabolites produced by some molds of *Aspergillus, Penicillium* and *Rhizopus* species, especially *A. flavus* and *A. paraciticus*. The clinical picture caused by aflatoxins in humans and animals is defined as "aflatoxicosis" (Rajarajan et al., 2021).

According to the color they give under ultraviolet (UV), aflatoxins; It consists of six main compounds: aflatoxin B1(AFB1), B2, G1, G2 and M1, M2. AFM1 and M2, known as milk toxin, are derivatives of AFB1 and B2 that are excreted in milk. The most toxic of aflatoxins is AFB1. The carcinogenic effect of aflatoxin M1 is 10 times lower than that of AFB1 (Jiang et al., 2021).

Aflatoxins are compounds that have toxic effects on humans and all animal species. Besides carcinogenic, mutagenic, teratogenic, hepatotoxic and immunosuppressive properties of aflatoxins, it has been reported that they are effective in the formation of kidney damage and various organ tumors (Rajarajan et al., 2021).

AFB1 has been defined as a "Class 1 carcinogen" and AFM1 has been defined as a Class 2B "probable human carcinogen" by the International Agency for Research on Cancer (IARC). In the classification made in 2002, AFM1 was included in the Class 1 list (IARC, 1993; IARC, 2002).

Due to its negative effects on human and animal health, legal regulations have been introduced for aflatoxins in many countries. The European Union (EU) Commission (EC, 2010) reported the maximum AFM1 level that can be found in milk and dairy products as 50 ng/kg. In the Turkish Food Codex Contaminants Regulation (TFC, 2011); the maximum limit of aflatoxin M1 in milk used in the production of raw milk, heat-treated milk and milk-based products has been determined as 0.050 µg/kg. This level has been reported as 0.025 µg/kg in infant formulas and follow-on formulas (including infant milk and follow-on milk).

The aim of this study is to investigate the presence and level of AFM1 in 72 infant formulas with 16 different brands offered for sale, to determine the estimated daily intakes, to determine the exposure to aflatoxin and to evaluate the data in terms of public health.

## Materials and Methods

### *Infant Formula Samples*

All the brands (n=16) of powdered infant formulae marketed in Türkiye (n=72) were collected from market and pharmacies. Samples from two production dates of each brand were compiled (total of 144 samples) within the same day of purchasing and stored in well-sealed containers. Tests were performed within the same week of purchase. While purchasing, production dates were checked.

### *AFM1 Detection by ELISA*

Biotech Aflatoxin M1 kit (R1408, Koon, Shanghai, China) was used for the quantitative analysis by ELISA. Sample preparation was done according to the kit instructions. Infant formulae powder (10 g) was weighed in a flask that was diluted to 100 mL with distilled water. Then, it was homogenized by stirring and warmed to 50 °C in a water bath for 30 min. A volume of 100 µL was used (Elaridi et al., 2019). The kit instructions were followed, whereby wells were coated with antibodies directed against anti-aflatoxin M1 antibodies, followed by the addition of anti-aflatoxin M1 antibodies, standards or sample solutions, enzyme conjugate, and substrate/chromogen. Finally, a quenching solution was added, which changed the color from blue to yellow that was measured spectrophotometrically at 450 nm. The analysis was conducted in duplicate. According to the AFM1 test protocol, the recovery rate in powdered milk was 95%. A standard curve for AFM1 was constructed with six points (0, 5, 10, 20, 40, 80 ng/kg). The limit of detection is 5 ng/L.

### *Aflatoxins Exposure*

The daily intake for each studied AFM1 was estimated considering the concentration of the metal obtained from the analysis of the samples, the average daily/weekly intake of the formula, and the average body weight (bw) for girls and boys separately. Daily doses were calculated using the infant's feed tables. The average bw was determined according to the child growth standards charts developed by WHO (FAO/WHO, 2008) considering the P95th percentile of the weight for girls and boys at 1st week (for the period of life of 0–2 weeks), 3rd week (for 2–4 weeks), 1st month (for 2 months), 4th month (for 4 months), 6-9 months, 9-12 months and 12-36 months (Bashiry et al., 2021, Demir et al., 2021). The daily intake for each formula was calculated by the following equation:

$$EDI = (X \times C) / BW$$

Where;

EDI: Is the daily estimated dietary intake of formulas expressed as (ng/kg bw/day)

X: Is the mean concentration average of total AFs levels in the formulas, expressed as ng/g

C: Is the consumption rate of formulas (g)

BW: Is the body weight expressed as kg.

### *Margin of Exposure Characterization for AFM1*

Carcinogenic and genotoxic compounds like aflatoxins have their risk assessment fittingly computed based on the Margin of Exposure (MOEs) apply to, which was estimated by basing that the Benchmark dose lower limit (BMDL) for aflatoxins 400 ng/kg bw/day by toxin exposure.

Food authorities identified the liver carcinogenicity of aflatoxins as the critical result of the risk assessment (EFSA, 2020); therefore, the BMDL confidence limit for a benchmark response of 10% (BMDL10) regarding the frequency of hepatocellular carcinomas (HCCs) in male rats was considered. A public health alarm is raised in instances where MOEs are less than 100.000 (JECFA, 2001; Adetunji et al., 2018; Kortei et al., 2021).

### *Statistical Analysis*

The AFM1 concentrations were calculated using regression analysis from the curves generated from the standards of AFM1 with Excel for Microsoft Windows

(version 16). One sample t-test was used to compare the means obtained at a 95% confidence interval and 5% level of significance. SPSS 22 (Chicago, USA) was used in the analysis of data. Deterministic risk assessment models were used; dietary exposure (Estimated Dietary Intake), MOE values, Average potency.

**Results and Discussion**

Milk and dairy products are among the foods that pose a risk in terms of aflatoxin. This is especially important for babies and children. This study was conducted to investigate the presence and level of AFM1 in 72 infant formulas with 16 different brands offered for sale, and to evaluate the data in terms of public health. Whether the level of AFM1 in 72 milk-based infant formulas sold in the market exceeds 0.025 µg/kg, which is the highest limit value specified for AFM1 in the Turkish Food Codex Contaminants Regulation, was examined by ELISA method and the analyzes were carried out. The limit set by the EU commission for AFM1 is; determined as 0.025 µg/kg in infant diet foods and follow-on milk for medical purposes (EC, 2010).

According to the analysis findings, AFM1 was detected in 35 (49%) of 72 samples examined. AFM1 values were determined as minimum 0, maximum 0.028 and mean 0.0136±0.010 µg/kg. As a result of the analysis, it was determined that there were 5 samples exceeding the maximum limit value (0.025 µg/kg) specified in milk-based infant formulas (Table 1). Different groups were taken into account when analyzing the samples. These groups are; Premature, hypoallegenic, 0-6 months, 6-9 months, 9-12 months and 1-3 years old. If it is necessary to evaluate within the groups, no sample exceeding the limit value determined by both the EU Commission and the Turkish Food Codex was found in the premature and hypoallergenic groups. So that; AFM1 was detected in only 2 (25%) of the premature samples and the mean AFM1 level was 0.0015±0.0006 µg/kg. In the hypoallergenic group, the mean AFM1 level was found to be 0.0017±0.0003 µg/kg, and AFM1 was detected in 6 (37.5%) of 16 different hypoallegenic infant formula samples. In hypoallergenic and premature infant formulas (24 samples), no sample exceeding national and international borders was found.

AFM1 was detected in 61% (11 samples) of 18 samples in the 0-6 months Infant formula group, and the highest values were measured as 0.027±0.001 µg/kg and 0.026±0.002 µg/kg. In the aforementioned group, it was determined that the AFM1 level exceeded the limit value specified in national and international standards in 2 samples. Among infant formulas, the AFM1 level in the 6–9-month group (10 samples) was found in the range of 0-0.0024 (µg/kg, and the average was 0.0019 (40% of the samples) µg/kg. In this group, no values exceeding the limits set by the standards were found

Among infant formulas, the AFM1 level in the 6–9-month group (10 samples) was found in the range of 0-0.0024 (µg/kg, and the average was 0.0019 (40% of the samples) µg/kg. In this group, no values exceeding the limits set by the standards were found. When the AFM1 levels of the 12-36 months (1-3 years) follow-up formulas were examined among the groups, AFM1 was detected in 8 (80%) of the 10 samples. The mean value of the toxin found was 0.0223±0.0048 µg/kg, and it was observed that two samples exceeded national and international standards. Considering all of the ınfant formulas obtained from different brands (72 samples) and evaluated in six different groups, AFM1 was detected in 35 (49%) of them. The difference between the groups was statistically significant ($P<0.05$).

AFM1 is a hydroxylated toxic metabolite of Aflatoxin B1 produced by *Aspergillus* species (Shundo et al., 2009). From a food standpoint, the riskiest group for humans to be exposed to AFM1 is milk and dairy products. The fact that babies, especially the premature and 0–6-month groups consume more milk compared to other age groups, increases the possibility of exposure to this toxin (Gürbay et al., 2006). In other months of development, milk consumption leaves its place to dairy products and foods prepared from milk. Consumption of milk-based infant foods on the market poses a serious risk to infants. In fact, a possible zoonotic disease raises a serious health risk such as Aflatoxicosis M1 (Wael et al., 2011).

There are a limited number of studies in the literature investigating the AFM1 level of milk-based infant formulas obtained from different brands. When these studies were evaluated, in a study investigating the presence and level of AFM1 in 20 different infant formulas using the ELISA method, AFM1 was detected in all of the samples. In this study conducted in Jordan, recorded AFM1 levels were found to be between 0.0165-0.1541 µg/kg, and 85% of the samples were reported to be above the limit value set by the EU commission (Omar, 2016). These values were found to be quite high when compared with our study findings.

In a different study, Alvito et al. (2010) investigated the presence and level of three different toxins (AFM1, AFB1 and OTA) in both milk-based and cereal-based infant foods. In the results of the study, the number of samples contaminated with AFM1 was recorded as 4 (26%) among the 15 samples, and it was stated that the AFM1 levels of the samples were in the range of 0.017-0.041 µg/kg. The study findings were determined to be quite high when compared with our study. The reason for this situation was thought to be due to the fact that countries use different techniques (risk of contamination) in the milk-based infant formula production process.

Aflatoxin problem in dairy products arises as a result of the presence of toxins in milk or milk powder and additives used in production, or the development of toxic *Aspergillus* species in these products in the stages after milking (Ağaoğlu et al., 2020). For molds that synthesize aflatoxin, 24-35°C temperature and 70% relative humidity are optimum growth conditions. The required temperature for toxin formation is 25-30°C. Food type and composition, water activity, ambient temperature, relative humidity, gases in the environment, especially atmospheric oxygen and carbon dioxide level, storage period, storage conditions and harvesting method are the factors that affect mold growth and toxin formation (Bulca and Bircan, 2013; Ağaoğlu et al., 2020).

In a study conducted in Lebanon, the presence and level of AFM1 was investigated using 84 infant formula ELISA method. Of the 84 infant formula samples analyzed, 74 (88%) were recorded as positive samples (0.0201±0.0013 µg/kg).

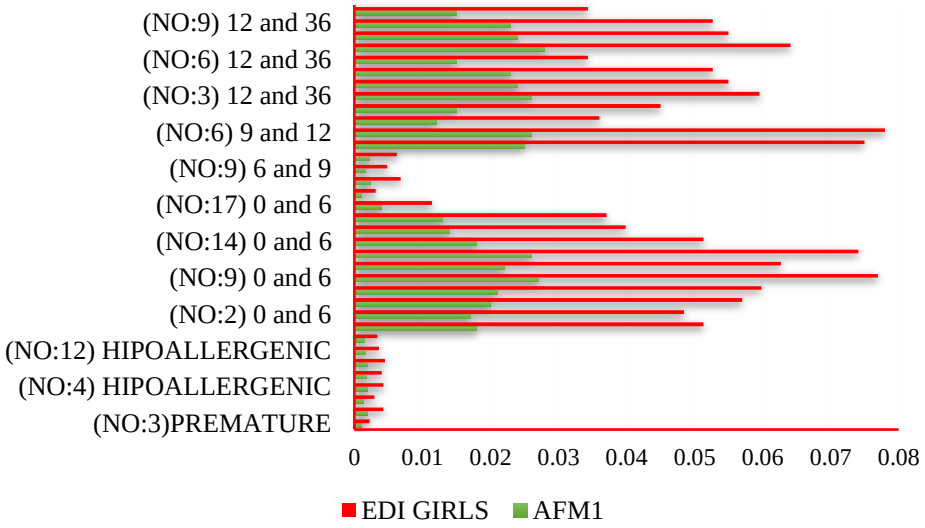



Figure 1a. Estimated daily intake (EDI) of AFM1 in all infant formulas

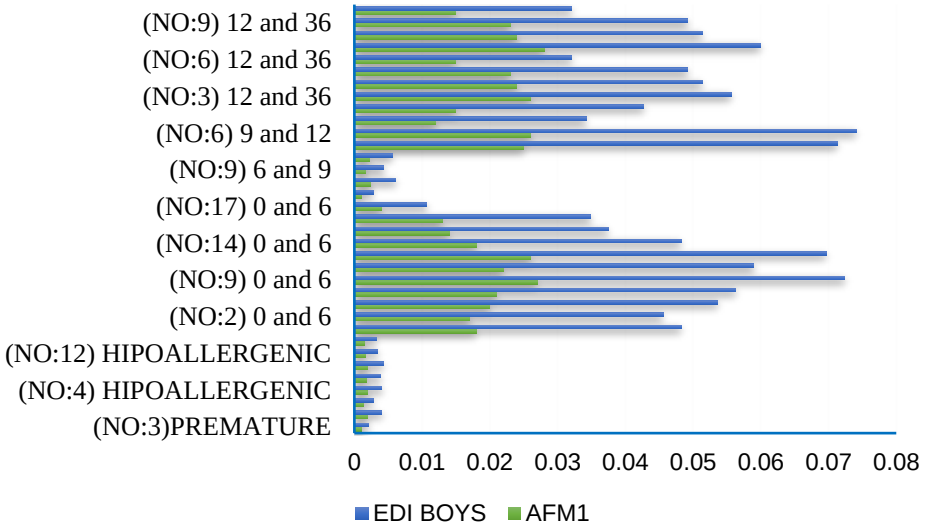
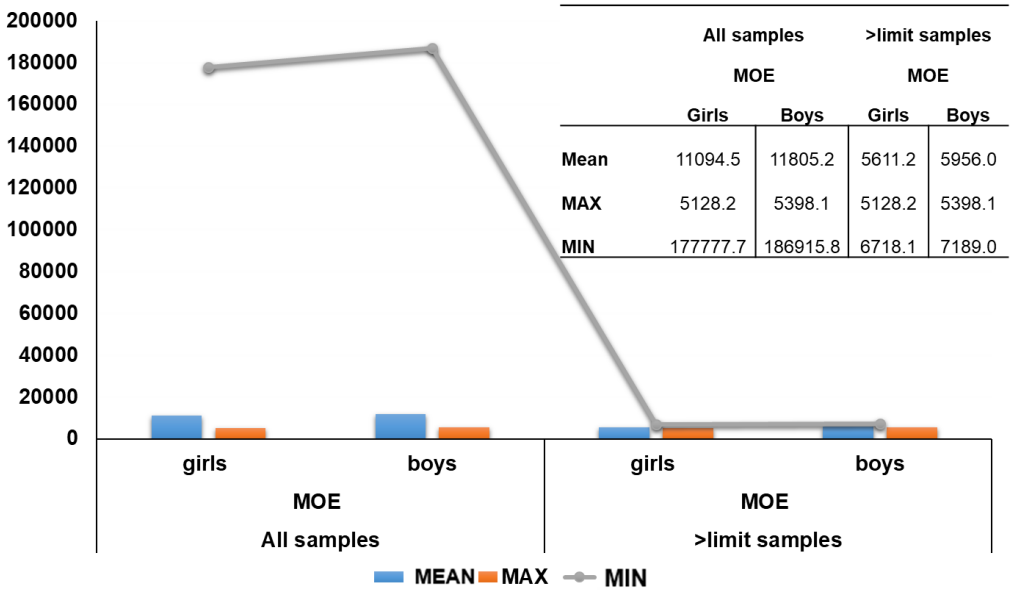



Figure 1b. Estimated daily intake (EDI) of AFM1 in all infant formulas




| | All samples MOE | | >limit samples MOE | |
|---|---|---|---|---|
| | Girls | Boys | Girls | Boys |
| Mean | 11094.5 | 11805.2 | 5611.2 | 5956.0 |
| MAX | 5128.2 | 5398.1 | 5128.2 | 5398.1 |
| MIN | 177777.7 | 186915.8 | 6718.1 | 7189.0 |

Figure 2. Margin of exposure characterization for AFM1 & Results

Table 1. AFM1 average concentration for all infant formula groups ± standard deviation

| Group no | Group name | Sample size (n) | Positive samples [n (%)] | Concentration range (µg/kg) | Average Mean ± SD | >limit |
|---|---|---|---|---|---|---|
| 1 | Premature (8) | 8 | 2 (25%) | 0-0.0019 | 0.0015±0.0006 | 0 |
| 2 | Hipoallergenic (16) | 16 | 6 (37.5%) | 0-0.0020 | 0.0017±0.0003 | 0 |
| 3 | 0 and 6 (18) | 18 | 11 (61%) | 0-0.027 | 0.0182±0.0064 | 2 |
| 4 | 6 and 9 (10) | 10 | 4 (40%) | 0-0.002 | 0.0019±0.0006 | 0 |
| 5 | 9 and 12 (10) | 10 | 4 (40%) | 0-0.026 | 0.0195±0.007 | 1 |
| 6 | 12 and 36 (10) | 10 | 8 (80%) | 0-0.028 | 0.0223±0.0048 | 2 |
| Total | | 72 | 35 (49%) | 0-0.028 | 0.0136±0.001 | 5 |

Among the positive samples, approximately 26 (31%) were stated to exceed the limit values of the EU commission from international standards (Elaridi et al., 2019). In a study conducted in China, 1207 milks were obtained from a factory that makes milk powder, which is a raw material for baby food. Collected Milk was subjected to AFM1 analysis on the basis of ELISA method. AFM1 positive was detected in 4.6% of the samples (56 samples). While the results were found to be above the American standards (0.05 µg/kg), they were reported to be below the national Chinese standards (62.5 ng/L) (Li et al., 2016). When the values were compared with the results of this study, it was seen that the amount of samples contaminated with AFM1 was lower. In addition, when compared with the American and Chinese standards, our study results showed compliance with these standards and it was seen that the mentioned standards did not exceed the limits. This means that the infant formulas offered for sale in Turkey do not carry any risk in terms of AFM1, comply with the standards and do not threaten public health.

In Italy, 2/185 (1%) ınfant formula samples included AFM1 (range 0.0118–0.0153 µg/kg), but at levels below the EC commission limit (Meucci et al., 2010). In Spain, AFM1 was recorded in 8/69 (12%) of infant formulas, with a mean concentration of 0.0031 µg/kg and a range of 0.0006-0.0116 µg/kg, and none exceeded the EC comission limit (Gómez et al., 2010). In Taiwan, no AFM1 was detected in 21 analyzed baby infant formula samples (Lin et al., 2004). In Greece, no kids milk sample had AFM1 level exceeding the EC limit (Tsakiris et al., 2013).

Aflatoxins exposure & Results and EDI values analyzed in six different groups are shown in Figure 1a-b. EDI values of AFM1 detected samples ranged from 0.001 to 0.028 ng/kg bw/day. The mean was determined as 0.014 ng/kg bw/day. When the results were evaluated, the riskiest group for both girls and boys was the 9- and 12-months group. The following group was 0 and 6 groups. In the literature, there are many studies investigating AFM1 levels of infant formulas and calculating EDI values.

In one of these studies, AFM1 level was determined in 520 milk samples collected in different seasons in Pakistan. While AFM1 was determined in 53% of the samples, EDI values were calculated as 0.22-5.45 ng/kg bw/day. (Ismail et al., 2016). In a different study, the level of AFM1 in milk collected in Serbia was investigated and the average EDI (in infants) value was determined as 2.65 ng/kg bw/day (Radonic et al., 2016). These values were found to be quite high when compared to our study.

The results obtained when looking at both tables; The EDI value of the group with the highest level of AFM1 is not high either. Because the daily intake doses of infants differ from each other according to the growth Percentil curve determined according to the nutrition chart and WHO. This increases the rate of exposure of infants to AFM1 at intake doses. Another difference between EDI values was that girls were more exposed than boys (Figure 1a-b).

On the other hand, another difference within the same group is the Brand difference. The height (EDI) in the same group and in the same percentile value is related to the brand quality. The Joint FAO/ WHO Expert Committee on Food Additives (JECFA) has not established a tolerable daily intake (TDI) for AFM1, but strongly recommended that the level of AFM1 should be "as low as reasonably achievable" (JECFA, 2001). Margin of exposure characterization reported that A public health alarm is raised in instances where MOEs are less than 100,000 (EFSA, 2020). The Margin of Exposure (MOE) for girls' and boys' values recorded were 11094.5 and 11805.2, respectively (Figure 2). MOE results obtained in a different study have been reported to mean high risk (total aflatoxins) for infants, children and adolescents (Kortei et al., 2021). Our results did not show a high risk of cancer for infants due to AFM1 exposure from infant formula consumption.

## Conclusion

Aflatoxin M1 (AFM1) contaminates milk and makes its consumption potentially dangerous. Infants are mostly at risk because they are typically fed as many as six and more times per day, which is indeed a disquieting concern. The incidence of aflatoxins in infant foods and consumption through babies' nutrition cause risk in any society and is hazardous for public health. Therefore, more limited control measures for preventing cereal-based ingredients' contamination for the manufacture of infant foods are compulsory, particularly in high-risk areas, along with close surveillance of aflatoxin levels in commercially present products.

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